Nauplius
The Journal of The Brazilian Crustacean Society

e-ISSN 2358-2936
www.scielo.br/nau
www.crustacea.org.br

Original Article

Light and electron microscopic studies of hemocytes in the endemic freshwater crab *Arcithelphusa cochleariformis* (Brachyura: Gecarcinucidae)

Pothera Veedu Anjali-Krishna[1]
Moorkoth Kunnath Smija[1]

[1] Department of Zoology Kannur University, Mananthavady Campus. Mananthavady 670 645, Wayanad, India.
E-mail: anjalikrishnapv14@gmail.com
E-mail: smijamkkunnath@gmail.com

ZOOBANK: http://zoobank.org/urn:lsid:zoobank.org:pub:FBF684F3-2277-49A9-A9F6-26C947CE894B

ABSTRACT

The morphological and structural characterization of hemocytes was made in the endemic freshwater crab, *Arcithelphusa cochleariformis* using light microscopy and transmission electron microscopy and the present study provides valuable information about the cell mediated immune responses of this freshwater crab. The ultrastructural studies show four types of hemocytes: agranulocyte, granulocyte I, granulocyte II and semi-granulocyte. Agranulocytes are oval to elongate and contain free ribosomes, RER cisternae and a few mitochondria with parallel cristae. They are mostly devoid of granules but rarely a few (1–3) small granules can be observed in their cytoplasm. Our results demonstrate that the circulating agranulocytes are phagocytic in function as evident from their prominent large bulbous pseudopodial projections. Granulocytes I are round or oval in shape, possess abundant granules of differing size, shape and electron densities and their cytoplasm contains mitochondria, free ribosomes, vacuoles and lysosomes. Granulocytes II are roughly spherical or round, display RER cisternae, mitochondria and large dense granules. Certain granulocyte II cells resemble autophagosomes and include materials at various stages of degeneration and multivesicular bodies, suggesting an autophagy function. Semi-granulocytes are round, oval, elongate or irregular cells with free ribosomes, RER cisternae, mitochondria, vesicles and lysosomes. They display lysosomal compartments with phagocytized materials and melanin deposition. We here suggest their possible role in the phagocytosis and melanization process.

KEYWORDS

Cell mediated immunity, Crustacea, Decapoda, granulocytes, ultrastructure

Corresponding Author
Moorkoth Kunnath Smija
smijamkkunnath@gmail.com

Submitted 09 May 2024
Accepted 22 December 2024
Published 13 October 2025

DOI 10.1590/2358-2936e20250556

INTRODUCTION

Comparable to other arthropods, crustaceans possess open vascular systems wherein hemocytes circulate throughout the hemolymph. Hemocytes, the primary components of the immune system perform innate, cellular and humoral reactions. Their role has been well recognized in vital defence reactions such as phagocytosis, prophenol oxidase activity, melanization, encapsulation and coagulation (Johansson et al., 2000; Matozzo and Marin, 2010). Moreover, they have pleiotropic non–immune functions like moulting, synthesis of hemocyanin and storage of nutrients (Vacca and Fingerman, 1983; Adachi et al., 2003).

Many categories of crustacean hemocytes have been recognized based on hemocyte properties or observation techniques (Hose et al., 1990). In various decapod crustaceans, the predominant types of hemocytes vary. In crayfish *Cherax quadricarinatus* (von Martens, 1868), granulocytes and semi-granulocytes are the major types of hemocytes, whereas agranulocytes are rare (Li et al., 2021). In mud crabs *Scylla paramamosain* Estampador, 1950, semi-granulocytes and agranulocytes are the predominant cell types (Zhou et al., 2018). In penaeid shrimps *Sicyonia ingentis* (Burkenroad, 1938) and *Penaeus californiensis* (Holmes, 1900), small-granule hemocytes are the major cell types and agranular and large-granule hemocytes are found in lesser numbers (Martin and Graves, 1985). Considerable variation is observed in the types and structure of hemocytes in various decapod crustaceans.

Information on the structure of hemocytes was extensively investigated by using optical microscopy and electron microscopy in many marine decapod species (Johansson et al., 2000; Mengal et al., 2023). However, very little information is available about the structural and functional details of hemocytes in freshwater crabs (Latha et al., 2017; Deyashi and Chakraborty, 2022). To the best of our knowledge, no previous study regarding identification of hemocytes was carried out in the Indian endemic freshwater crab, *Arcithelphusa cochleariformis* Pati and Suda Devi, 2015. Therefore, the present study aims to investigate the circulating hemocytes of *A. cochleariformis*, using light microscopy and transmission electron microscopy. The information generated from the present study provides structural and functional details of circulating hemocytes of *A. cochleariformis* and the data can be used to design various new methods to elucidate the freshwater crab immune system.

MATERIALS AND METHODS

Adult crabs (intermoult stage) of carapace width 3.5–4.0 cm (n=4) were captured from the arecanut plantations of Ondayngadi, Mananthavady in Wayanad district Kerala, India during the period April to May 2022. The crabs were acclimated to the laboratory conditions for three days. Hemolymph (2ml) was obtained from the 4th walking leg with the help of a sterile needle and was transferred to an Eppendorf vial containing 3% glutaraldehyde in 0.1 M sodium cacodylate buffer as fixative.

The fixation was done for two hours and the sample was then centrifuged at 1000 rpm for five minutes. Further, fixation of the pellets was carried out in 3% glutaraldehyde, and they were washed in buffer, post fixed in 1% osmium tetroxide and washed in buffer again. The double fixation enhances staining contrast, provides reduced distortion and fixes fine structural details, appropriate for animal materials (modified from Schrand et al., 2010). This double fixation process provides stability throughout dehydration, embedding, and electron exposure (Schrand et al., 2010). The dehydration was carried out by using an ascending graded alcohol series (50%–100%) and clearing was done in propylene oxide. For infiltration, an ascending series of propylene oxide and epoxy resin mix was used, and then later, the tissue was embedded in 100% epoxy resin in silicon rubber molds.

The embedded mold was placed in an incubator at 60 ^{0}C for 48 hrs and blocks were cooled and sectioned. Semi-thin sections (One-micron thick) were prepared on an ultra-microtome (Leica ultra cut UCT) using a glass knife and stained with toluidine blue. Ultra-thin sections (less than100 nm) were prepared with the same ultra-microtome and with an attached diamond knife (Diatome). Ultra-thin sections were supported on copper grids and double stained with Reynold's solution (uranyl acetate and lead citrate) (Schrand et al., 2010). Sections were viewed and photographed

in a Philips Tecnai T12 Spirit transmission electron microscope, and analyzed for fine structural details.

The different types of hemocyte were counted from the semi-thin sections. The number of each cell type was totalled from four visual areas to calculate the percentage of different hemocytes. The measurements of hemocytes were carried out by using imaging software (Nikon NIS Elements) of the Nikon Eclipse Ni research microscope (China).

RESULTS

Light microscopy

Light microscopic analyses revealed four hemocyte types – granulocyte I, granulocyte II, agranulocytes and semi-granulocytes on the basis of the presence or absence of cytoplasmic granules. Granulocyte I (29%) nuclei possess thin patches of chromatin and cytoplasm contains abundant, intensely stained large granules distributed around the nucleus. In Granulocyte II (35%), intensely stained large granules fully fill the cytoplasm or sometimes, they completely obscure the nucleus. Agranulocytes (19%) were characterized by large nuclei and their cytoplasm mostly devoid of granules. Semi-granulocytes (17%) have round to oval nuclei with thin patches of chromatin attached to the nuclear membrane and the cytoplasm encompasses a few small and large granules (Fig. 1, Tab. 1).

Transmission electron microscopy (TEM)

Ultrastructural analyses by TEM confirmed the four cell types – agranulocyte (Ag), granulocyte I (GI), granulocyte II (GII), semi-granulocyte (Sgc) (Fig. 2).

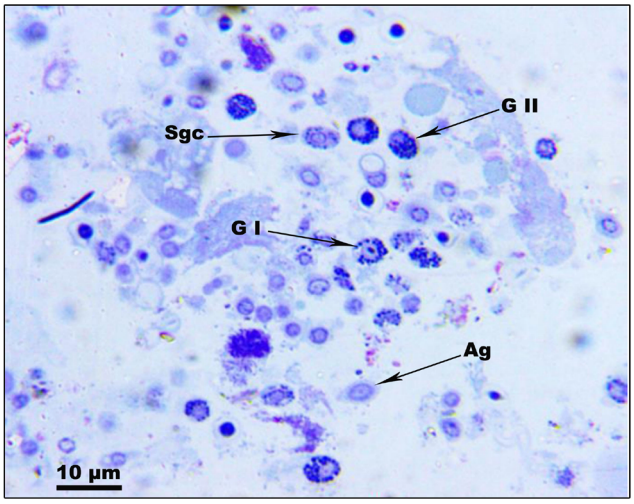

Figure 1. Light micrograph of circulating hemocytes in the hemolymph of the freshwater crab, *Arcithelphusa cochleariformis*. Abbreviations: Ag, agranulocyte; GI, granulocyte I; GII, granulocyte II; Sgc, semi-granulocyte. Scale bar = 10 µm.

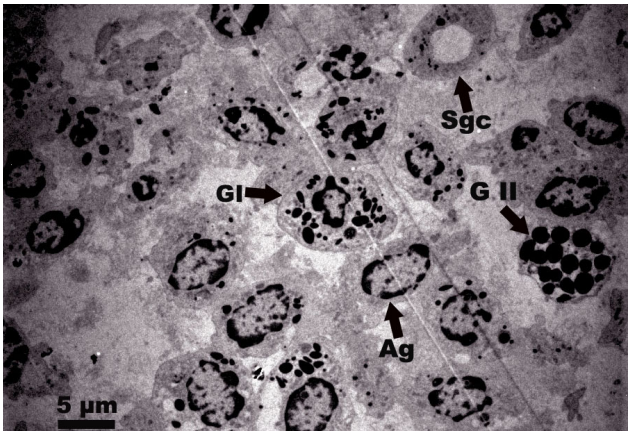

Figure 2. Ultrastructure of circulating hemocytes in the hemolymph of the freshwater crab, *Arcithelphusa cochleariformis*. Abbreviations: Ag, agranulocyte; GI, granulocyte I; GII, granulocyte II; Sgc, semi-granulocyte. Scale bar = 5 µm

Table 1. Types of hemocytes in the hemolymph of the freshwater crab, *Arcithelphusa cochleariformis*.

Parameter	Agranulocyte (Ag)	Granulocyte I (GI)	Granulocyte II (GII)	Semigranulocyte (Sgc)
Cell size (µm)	5.66×3.26 –11.80×7.60	8.92×6.30 –13.3×9.66	3.83×2.50 –10.33×8.66	6.2×4.13 –10.70×6.88
Nucleus size (µm)	1.61×0.92 –5.89×4.5	4.15×4.50 –5.33×5.84	2.34×2.83 –4.66×4.88	2.90×2.88 –5.66×4.88
Mean Nucleocytoplasmic ratio (NPR)	0.61±0.29	0.54±0.43	0.56±0.37	0.71±0.38
Average number of granules/cell	(2)	(44)	(26)	(14)

Agranulocytes

Hemocytes are oval to elongate cells (5.66 × 3.26–11.8 × 7.6 µm). The nucleus (1.61 × 0.92–5.89 × 4.50 µm) is round to oval, occupying most of the cytoplasmic space and nucleocytoplasmic ratio (NPR) is 0.61 ± 0.29. The large nuclei possess an even and discrete nuclear envelope containing dense heterochromatin adhered to the inner nuclear membrane and a marginal nucleolus (Fig. 3A). They usually lack cytoplasmic granules but occasionally one or three granules were noted (0.3–0.4µm). Numerous free ribosomes, RER cisternae, and a few mitochondria with parallel cristae were contained in the cytoplasm. RER cisternae are concentrated towards the perinuclear region. Vacuoles were observable in various sizes and shapes, mostly towards the periphery of the cell and containing moderately electron dense and flocculent materials. These cells are able to emit bulbous pseudopodial projections (Fig. 3B). The electron–lucent small vesicles appeared inside the pseudopodia and are considerably less frequent. The release of electron lucent contents of the vesicles out of the membrane is visible in those regions where pseudopodia are emitted (Fig.3A).

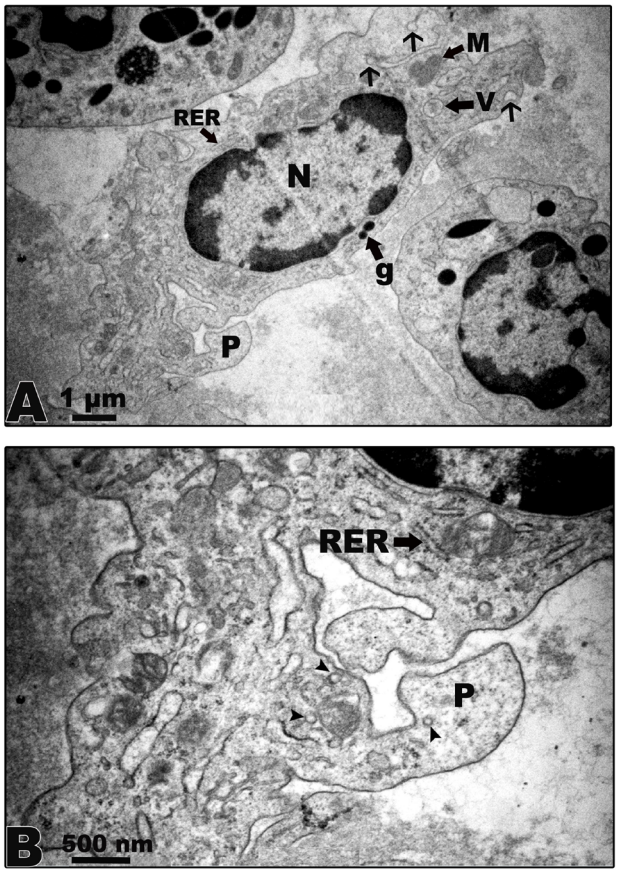

Figure 3. Transmission electron micrographs of agranulocytes in the hemolymph of the freshwater crab, *Arcithelphusa cochleariformis*. **A,** Ultrastructure of agranulocyte showing oval nucleus and cytoplasm with a single electron dense granule; **B,** Prominent bulbous pseudopodial extensions in agranulocyte (figure 3B is higher magnification of figure 3A). Abbreviations: g, electron dense granule; M, mitochondria; N, nucleus; RER, rough endoplasmic reticulum cisternae; P, pseudopodia; V, vacuole; upward arrow indicates secretion of electron lucent vesicles out of the plasma membrane; arrow head indicates small electron lucent vesicles. Scale bars: **A** = 1 µm; **B** = 500 nm.

Different granule types in the hemocytes

Based on the size, shape, and density, three granule types were identified in the hemocytes, which include type I, type II, and type III (Fig. 4A–C).

Type 1

Small to large (0.61 × 0.30–1.83 × 1.33µm), round, kidney-shaped, oval, elongate, tear, rod–shaped with homogeneous dense matrix. Smaller type 1 granules were usually found in GI, GII and Sgc. Dominant in GII.

Type 2

They are round, oval or elongate (0.92 × 0.46–2.16 × 0.84 µm). They consist of an electron–lucent matrix with a definite membrane. Mainly found in GI and Sgc.

Type 3

Oval, elongate, round granules (0.33 × 0.28–1.23 × 0.66 µm). They have mild electron lucent periphery. Frequently occur in GI and Sgc.

Granulocytes I

Typically, oval to round cells (8.92 × 6.30–13.3 × 9.66µm) with small, centric, or eccentric nuclei. Nucleoli (1–2) are peripheral in position. They exhibited low NPR when compared to that of Ag (0.54 ± 0.43). Many polymorphic granules were observed within the cytoplasm. The cytoplasm contains RER cisternae, free ribosomes, and mitochondria with parallel cristae. RER cisternae mostly seen surrounding the nucleus (Fig. 5A, B). Mitochondria were numerous and mostly round or elongate, dispersed in between polymorphic granules (Fig. 5B). A few vesicles of SER were observed towards the periphery of these cells.

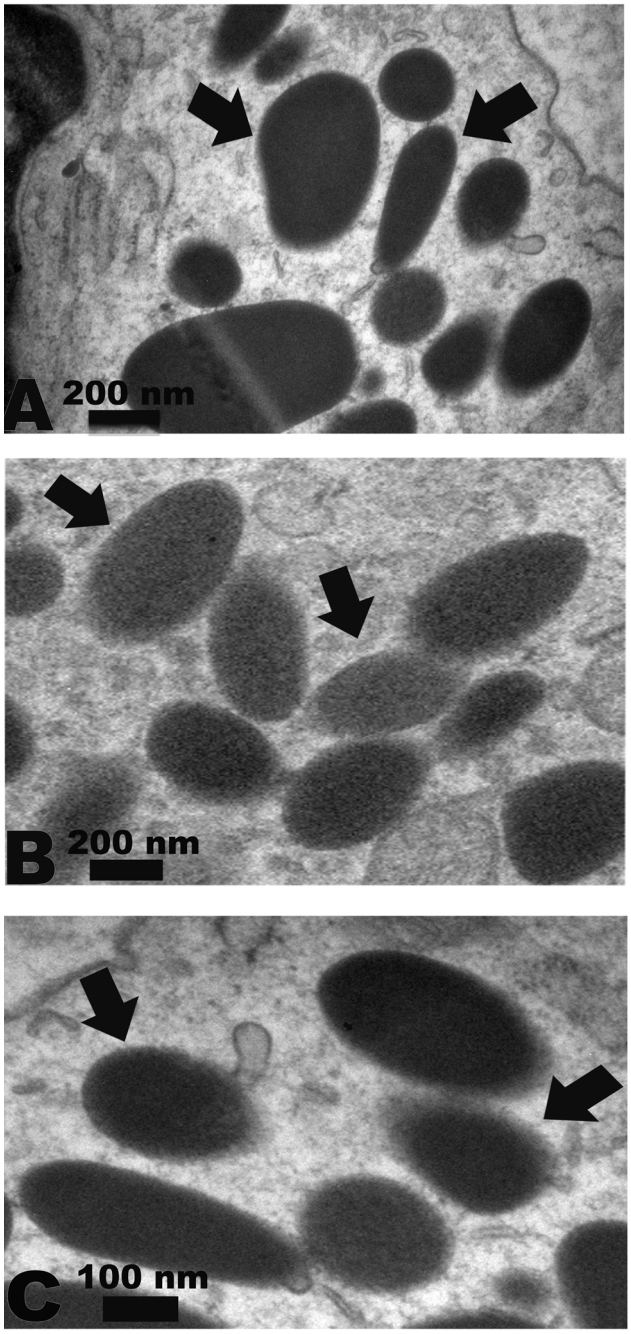

Figure 4. Electron micrographs of granule types in the hemocytes of the freshwater crab, *Arcithelphusa cochleariformis*. **A,** Type 1 granules in the cytoplasm of granulocyte I; **B,** Type 2 granules in the cytoplasm of granulocyte I; **C,** Type 3 granules in the cytoplasm of granulocyte I (figure 4C showing part of figure 4A). Arrows indicate type 1 granules in figure A, type 2 granules in figure B and type 3 granules in figure C. Scale bars: **A**–**B** = 200 nm; **C** = 100 nm.

Small pseudopodia or filopodia like projections are observed on the granulocyte I plasma membrane. Another observation is the presence of large lysosomal bodies in the cytoplasm.

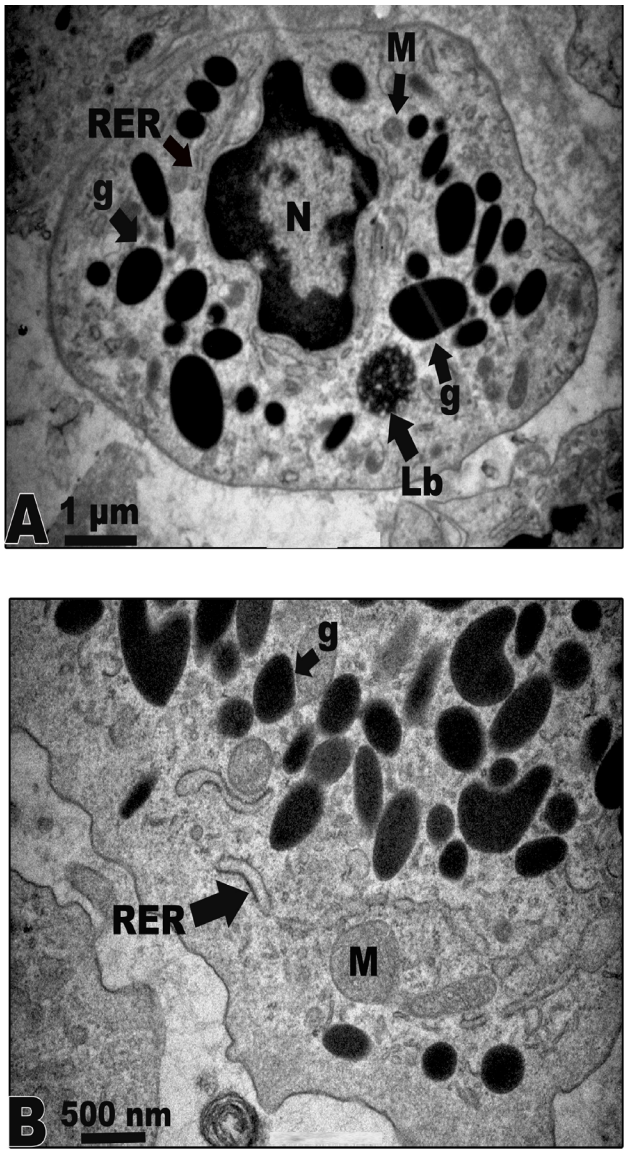

Figure 5. Fine structural details of granulocyte I in the hemolymph of the freshwater crab, *Arcithelphusa cochleariformis*. **A,** Granulocyte I with nucleus and cytoplasmic organelles; **B,** Granulocyte I with electron dense and electron lucent granules. Abbreviations: M, mitochondria; N, nucleus; RER, rough endoplasmic reticulum cisternae; g, granule; Lb, lysosomal body. Scale bars: **A** = 1 µm; **B** = 500 nm.

Granulocytes II

Roughly spherical or round cells (3.83 × 3.5–10.33 × 8.66 µm) containing mostly centric or rarely eccentric nuclei (2.34 × 2.83–4.66 × 4.88 µm). Roughly round or kidney shaped nucleus with large patches of chromatin on the inside of the nuclear membrane and one or two nucleoli appear peripherally (Fig. 6A). The NPR was found to be 0.56 ± 0.37. The cytoplasm encompassed RER cisternae, mitochondria and

dense granules. Mostly, large round or oval electron dense granules predominate in the cytoplasm of GII (Fig. 6B). Small and large vesicles contain electron dense flocculent material. These cells contain granules with electron dense filling. The plasma membrane of GII was also characterized by small pseudopodial projections. Certain GII cells had no granules in their cytoplasm and show autophagosomes containing materials at various levels of degeneration and multivesicular bodies towards the periphery of cells (Fig. 6 C, 7). These cells exhibit certain specific ultrastructural features like excessive condensation of the chromatin, noteworthy vacuole formation in the cytoplasm, and breakdown of the contents of the cytoplasm.

Semi-granulocytes

They were round, oval, elongate or irregular (6.2 × 4.13–10.7 × 6.88) with round or irregular shaped eccentric nuclei (2.9×2.88–5.66×4.88µm). Electron

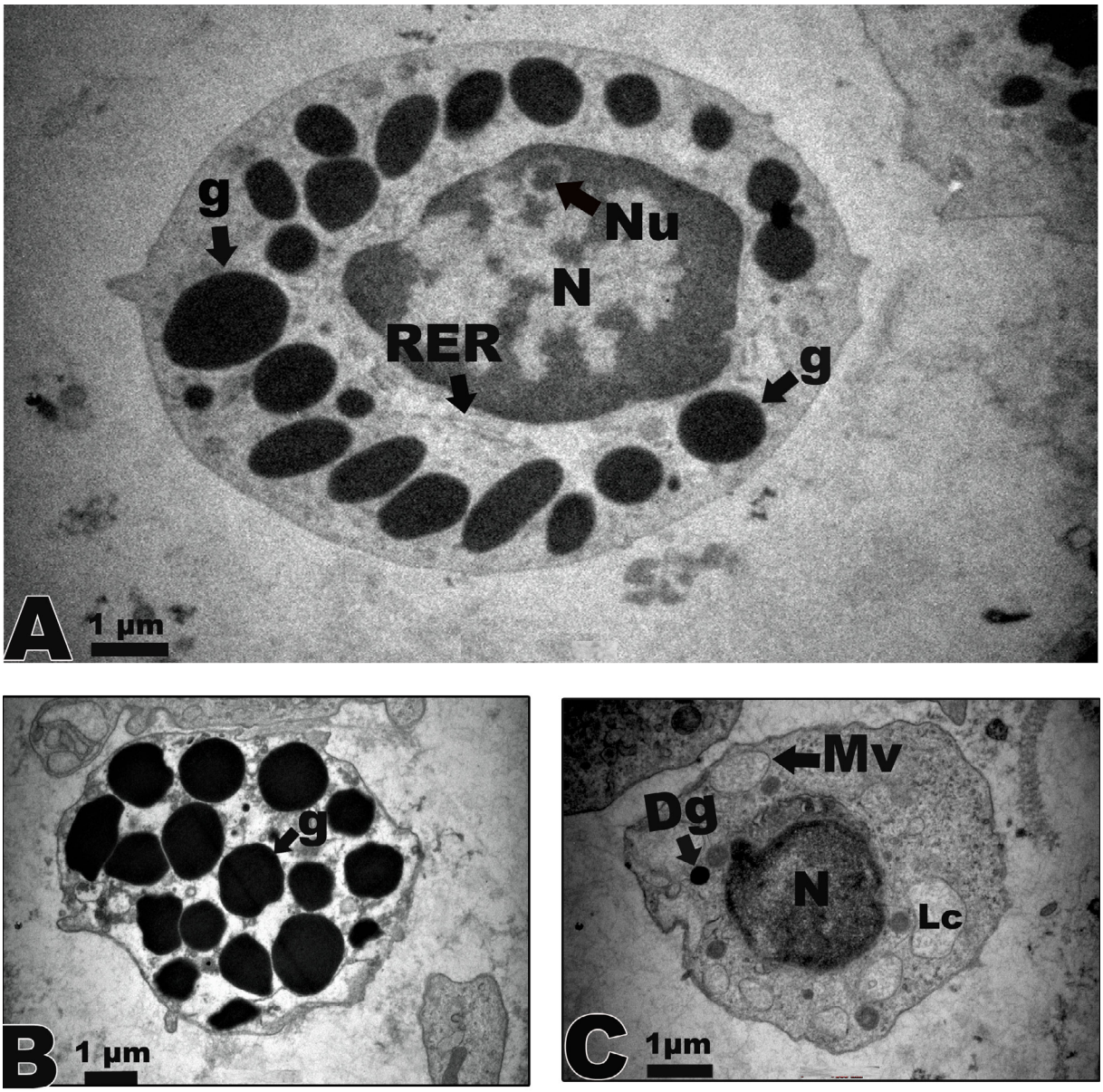

Figure 6. Electron micrographs of granulocyte II in the hemolymph of the freshwater crab, *Arcithelphusa cochleariformis*. **A,** Granulocyte II with nucleus and small pseudopodia; **B,** Granulocyte II with large electron dense granules completely obscuring the cytoplasm; **C,** Granulocyte II with multivesicular bodies and lysosomal compartments in the cytoplasm of GII. Abbreviations: Dg, dense granule; Lc, lysosomal compartment; N, nucleus; Nu, nucleolus; g, granule; RER, rough endoplasmic reticulum cisternae; Mv, multi vesicular body. Scale bars: **A**–**C** = 1 µm.

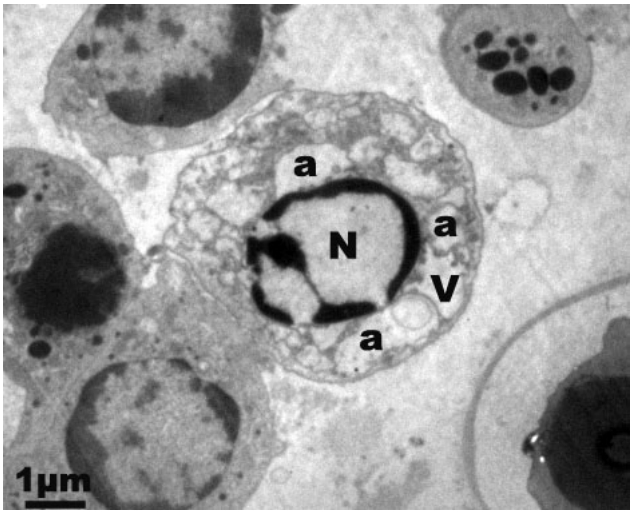

Figure 7. Detection of autophagosomes in granulocyte II of the freshwater crab, *Arcithelphusa cochleariformis* (figure 7 is an inset of figure 9). Abbreviations: a, autophagosome; N, nucleus; V, vacuole. Scale bar = 1 µm.

dense heterochromatin lines the interior of the nuclear membrane (Fig. 8A). The average NPR is high (0.71 ± 0.38). Several small electron dense (type I) granules were unequally distributed throughout the cytoplasm (Fig. 8B). Additionally, type 2 and 3 granules are also visible and the number of granules in Sgc ranges from 8–12. Free ribosomes, RER cisternae, mitochondria, SER vesicles and lysosomes are present in the cytoplasm. Several lysosomal compartments could be observed within the cytoplasm (Fig. 8 B). These cells possess endocytic vesicles with ingested materials. They show small pseudopodial projections around the plasma membrane and melanin deposition is visible in the cytoplasm. Certain semi-granulocytes that have phagocytized foreign substances appear to form close associations with other semi-granulocytes and granulocytes and developing dense aggregations, probably initiating nodule formation (Fig. 9).

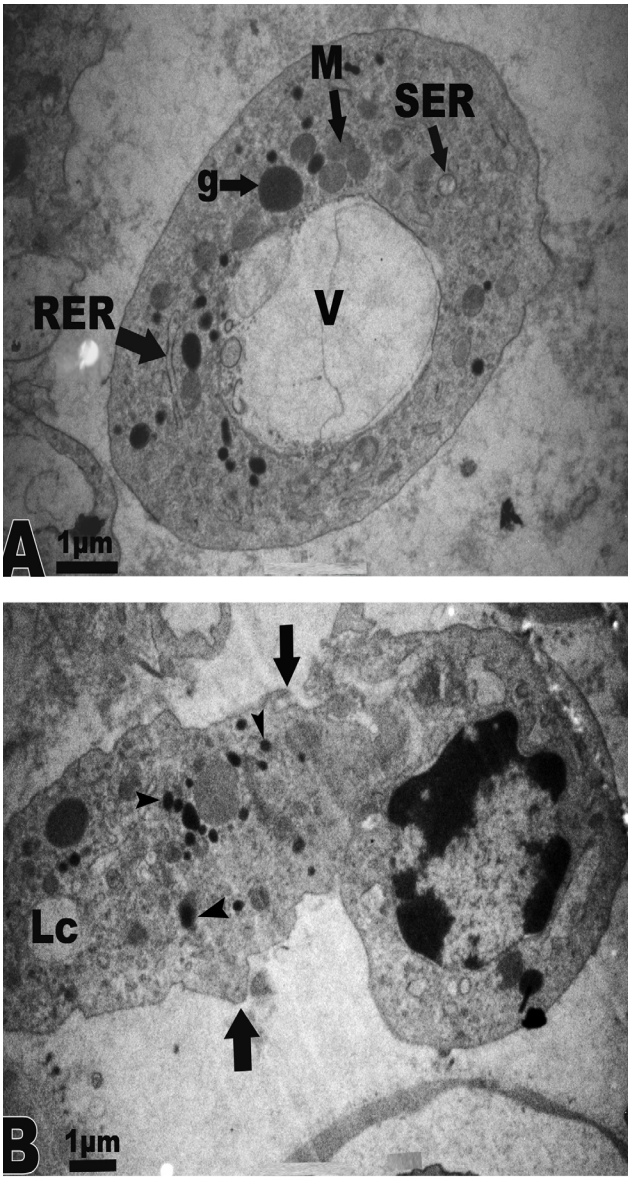

Figure 8. Electron micrographs of semi-granulocytes in the hemolymph of the freshwater crab, *Arcithelphusa cochleariformis*. **A,** Semi-granulocyte with cytoplasmic organelles and a large vacuole; **B,** Semi-granulocyte with small electron dense granules and lysosomal compartments. Abbreviations: g, granule; Lc, lysosomal compartment; M, mitochondria; RER, rough endoplasmic reticulum cisternae; SER, smooth endoplasmic reticulum vesicle; V, vacuole; arrow heads indicate small electron dense granules; arrow indicates small pseudopodia in semi-granulocyte. Scale bars: **A**–**B** = 1 µm.

DISCUSSION

For crustaceans in general, the most widely accepted idea is that there are at least three types of hemocytes. Most criteria are exclusively dependent on the granule appearance in the cytoplasm and the hemolymph of crustaceans show three different hemocyte types (Bauchau, 1980). Different authors have emphasized the importance of various techniques and assays which give information about cell populations and their functions (Davey and Kell, 1996). In the present study, we have used both light and electron microscopic information to classify the circulating hemocytes of *A. cochleariformis* and distinguished four cell types: agranulocytes, granulocytes I, granulocytes II, and semi–granulocytes. The present observations are in

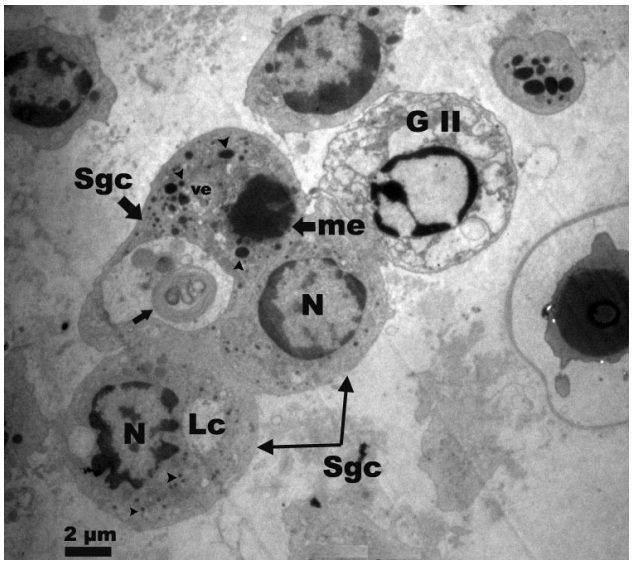

Figure 9. Electron micrograph of semi-granulocytes phagocytosing foreign particles and forming aggregations noted in the hemolymph of the freshwater crab, *Arcithelphusa cochleariformis*. Abbreviations: GII, granulocyte II; Lc, lysosomal compartment; me, melanization event; N, nucleus; Sgc, semigranulocyte; Ve, vesicle; arrow heads indicate small and large electron dense granules in Sgc; arrow indicates Sgc phagocytosing foreign materials. Scale bar = 2 µm.

agreement with Deyashi and Chakraborty (2022) who identified three hemocyte types, namely hyalinocytes, small granule hemocytes and large granule hemocytes in the freshwater crab, *Varuna litterata* (Fabricius, 1798). Martin and Graves (1985) classified three hemocyte types in penaeid shrimps which include large granular, small granular and agranular cells. However, in the freshwater crab, *Travancoriana schirnerae* Bott, 1969, Latha et al. (2017) identified three types which are agranulocytes, granulocytes I and granulocytes II.

In the present study, agranulocytes of *A. cochleariformis*, are mostly void of granules, and their ultrastructural features were analogous to that of hyaline cells, identified in other decapod crustaceans (Matozzo and Marin, 2010; Wentao et al., 2017). They project large bulbous pseudopodial projections, indicating their role in phagocytosis. In the current study, it is obvious that the less granular agranulocytes play a major role as phagocytes, as they account for 32% of the total hemocyte population. Similarly, in the freshwater crab *V. litterata*, various cytoplasmic organelles were copiously present in the hyalinocytes and the presence of pseudopodial extensions in hyalinocytes indicate phagocytic function (Deyashi and Chakraborty, 2022). In *Carcinus maenas* (Linnaeus, 1758), hyaline cells act as the chief phagocytic cells (Söderhäll et al., 1986). In *Carcinus. aestuarii* Nardo, 1847, granulocytes and hyalinocytes appeared in round or amoeboid shapes and they project pseudopodial projections from their membrane. Wu et al. (2019) reported hyaline cells as dominant phagocytes in horseshoe crabs, *Trachypleus tridentatus* (Leach, 1819) and *Carcinoscorpius rotundicauda* (Latreille, 1802). When we broaden comparisons to insect species, phagocytosis is primarily mediated by plasmatocytes, cells which are rich in cytoplasmic organelles and numerous membrane protrusions (Bruno et al., 2023). By contrast, in many lepidopteran species, phagocytic activity was primarily executed by granulocytes (Tojo et al., 2000; Wu et al., 2015), while in coleopterans, equally oenocytoids and granulocytes perform phagocytic function (Giulianini et al., 2003). Accordingly, it appears that both in crustaceans and insects, different types of hemocytes, like agranulocytes and granulocytes, are specialized for performing phagocytic function. Studies on decapod crustacean hemocytes have shown that hyalinocytes are predominantly involved in phagocytosis (Matozzo and Marin, 2010; Mengal et al., 2023). In invertebrates, phagocytes perform a significant function in the innate immune responses (Jiang et al., 2016). Furthermore, phagocytosis seems an early extremely preserved immunological process in all eukaryotes, especially in insects and crustaceans (Bouallegui, 2021; Bruno et al., 2023) to combat against invading microorganisms (Liu et al., 2020). In crustaceans, phagocytosis by hemocytes has been observed as a vital defence reaction for the host towards microbial organisms like bacteria and viruses (Liu et al., 2020).

In the current study, two granulocyte types were observable and their cytoplasm was dominated by large electron-dense granules, signifying their functional similarity. The present observations are in agreement with Deyashi and Chakraborty (2022), who identified two types of large granule hemocytes in the freshwater crab, *V. litterata*. The structure of nucleus, cytoplasmic organelles and the distribution of large electron-dense granules were very similar between granulocytes of *A. cochleariformis* and *V. litterata*.

Comparably, Latha et al. (2017) have reported the occurrence of granulocytes I and II in the freshwater crab, *T. schirnerae*. In *T. schirnerae*, granulocytes II were the conspicuous hemocytes and they possessed noticeably large, electron dense type I granules. In some granulocyte II cells, granules were totally obscuring the nucleus. In our TEM observations of GII, we have also noticed similar features. We have noticed the abundance of RER, free ribosomes, and mitochondria in GI suggesting their involvement in metabolic and synthetic activity (Laxmilatha and Laxminarayana, 2004; Latha et al., 2017). According to our observations, large lysosomal bodies are visible inside the cytoplasm of GI. Many studies on hemocytes of marine invertebrates like crabs and oysters, report lysozymes in their hemocytes (Cho and Jeong, 2005; Jia et al., 2017; Wang et al., 2017). In horseshoe crabs, granular cells appear with greater lysosomal activity than that of hyaline cells (Wu et al., 2019). It was noted that in the green-lipped mussel *Perna viridis* (Linnaeus, 1758), granular cells showed greater lysosomal activity than that of other types of hemocytes (Wang et al., 2012). Lu et al. (2021) discovered high phagocytic activity and lysosomes in granulocytes of the Hong Kong oyster, *Crassostrea hongkongensis* Lam and Morton, 2003. Cytosolic lysosomes release different enzymes in vesicles which can non-specifically kill microorganisms (Carballal et al., 1997; Donaghy et al., 2009). Generally, in crustaceans, granular hemocytes play an important role in encapsulation, storage and release of prophenoloxidase (proPO) and cytotoxicity, with a restricted role in phagocytosis (Johansson et al., 2000; Vogan and Rowley, 2002). It is possible that GI in *A. cochleariformis* may perform synthesis and storage of enzymes required for the immune reactions against foreign particles and they have limited phagocytic function.

In the present study, some morphological differences were observed in certain GII. They had intense cytoplasmic vacuolization and displayed autophagosomes including materials at various phases of degeneration, suggesting phagocytosis and autophagy processes, respectively. Similarly, studies by Fiorotti et al. (2019) in the tick, *Rhipicephalus microplus* (Canestrini, 1888) showed cytoplasmic vacuolization in plasmatocytes from fungus infected ticks. In addition, the fungus infected tick's granulocytes exhibited seemingly less granules, vacuolization and autophagosomes with materials at different stages of degradation. The structural changes of plasmatocytes and granulocytes possibly indicate phagocytosis which may proceed to cell death, as previously described by Sharma et al. (2008), Shaurub et al. (2014) and Fiorotti et al. (2019). In invertebrates, autophagy appears to be a crucial process during pathogen-host cell interaction (Picot et al., 2019). In insects, exposure to pathogens like microbial organisms and parasites in hemocytes can activate cell mediated immunity (Rosales, 2017). Similar observation has been reported in molluscs; with ultrastructural studies in Pacific oyster hemocytes demonstrating autophagosomes when they are exposed to an autophagy inducer molecule (Picot et al., 2019). In our study, definite ultrastructural features associated with autophagy were observed, including highly condensed chromatin, considerable vacuolization of the cytoplasm, and dilapidation of the cytosolic materials. Similar observations were reported by Picot et al. (2019) in *Crassostrea gigas* (Thunberg, 1793) hemocytes, exposed to various autophagy inducer and inhibitor molecules. Herein, our observations demonstrate autophagosomes in granulocytes II, and which may give an impression of possible prior exposure to foreign materials like pathogens by hemocytes. Accordingly, in freshwater crabs, autophagy may play a significant role in the protection against potential pathogens and other foreign materials.

In *A. cochleariformis*, semi-granulocytes exhibit considerable morphological changes in cell shape such as round, oval, elongate or irregular. Ultrastructure of semi-granulocytes revealed numerous cytoplasmic organelles like RER, ribosomes, SER, mitochondria, vacuoles, lysosomes and numerous small dense granules throughout the cytoplasm. Similar small dense cytoplasmic granules have been reported in the semi-granulocytes of other crustacean species such as *V. litterata* (Deyashi and Chakraborty, 2022), *Penaeus merguiensis* De Man, 1888 (Wang et al., 2002), *C. quadricarinatus* (Li et al., 2021) and *C. aestuarii* (Matozzo and Marin, 2010). In the present study, semi-granulocytes show several lysosomal compartments and possess endocytic vesicles with ingested materials. We can imagine a multifunctional role performed by the Sgc because they appear to be

involved in aggregation with other hemocytes, which is a prerequisite for nodule formation in cellular immunity, melanization and phagocytosis in addition to lysosomal activity. Interestingly, we can infer that the morphological and structural variations in the Sgc, may indicate innate immunological reactions in response to foreign particles, possibly a microbial attack. Similarly, Salem et al. (2014) reported that in the insect, *Galleria mellonella* (Linnaeus, 1758) after an infection with roundworm, *Steinernema carpocapsae* (Weiser, 1955), induced considerable structural changes in the hemocytes. The granular contents swell, giving a largely vacuolated appearance to cells. Hemocytes that engulf microbial organisms and foreign materials, adhere to each other, and develop aggregations. These unformed accretions may then be surrounded by other hemocytes, or by hemocytes that may be freed from the aggregations. Bruno et al. (2023) analysed morphological changes in hemocytes after bacterial attack in black soldier fly larvae, *Hermetia illucens* (Linnaeus, 1758). They detected plasmatocytes with phagolysosomes containing degraded bacteria and apoptotic cells with clearly recognizable cytoplasmic vacuolization and chromatin condensation. As mentioned above, Sgc adhere to each other, forming aggregation with other hemocytes like GII and Ag. In addition, in this process, GII demonstrate degranulation with prominent cytoplasmic vacuolization. The presence of GII and Ag close to Sgc, may indicate their active involvement in nodulation. We were not able to distinguish the roles of GII and Ag; but we may assume that GII may first release granules to chemo-attract Sgc and Ag towards the foreign materials. Comparably, the type of hemocytes involved in encapsulation and melanization is well studied in insects. Generally, in insects, granulocytes mediate first and depict degranulation to chemo-attract plasmatocytes towards the foreign agent, which are gradually surrounded by these hemocytes, becoming multilayered and eventually melanized (Bruno et al., 2023).

CONCLUSION

To conclude, we have identified four types of hemocytes in the endemic freshwater crab, *A. cochleariformis*. The morphological and structural differences were observed in all the four types of hemocytes. The granules appear to constitute a fundamental aspect of hemocyte morphology and physiology. Both GI and GII were rich in large granules; GI display lysosomes and that may indicate the presence of enzymes associated with the digestion of exogenous particles. GII exhibit intense cytoplasmic vacuolization and autophagosomes with contents at various phases of dilapidation and this is an unusual feature that was observed for the first time in freshwater crab hemocytes. These features highlight the importance of GII in phagocytosis and autophagy. It has been found that the presence of prominent bulbous pseudopodial projections of Ag may indicate phagocytosis function. In our study, Sgc is described as an important group in cell mediated immunity because it appears to involve in aggregation which is a prerequisite for nodule formation, melanization and phagocytosis in addition to lysosomal activity. Finally, we feel confident in stating that ultrastructural features of circulating hemocytes in the hemolymph of *A. cochleariformis* show significant difference and they perform specific functions as a part of cell mediated immunity. Moreover, the immune response of freshwater crabs appears very complex and studies on the structure of hematopoietic tissue and hematopoietic stem cells, the type and origin of antimicrobial molecules, and interaction and function of immune factors should be the focus of future research.

ACKNOWLEDGEMENTS

The authors heartily acknowledge Dr Anna B Pulimood, Professor, Christian Medical College (CMC) Vellore, Tamil Nadu, India for providing access to the transmission electron microscope facility. We greatly acknowledge Ms. A. Bascal Rita and Mr. C. Gunasekaran, CMC Vellore for their technical help in transmission electron microscopy. The authors greatly appreciate the love and support given by the staff of the electron microscopy lab.

REFERENCES

Adachi K; Hirata T; Nishioka T and Sakaguchi M 2003. Hemocyte components in crustaceans convert hemocyanin into a phenoloxidase-like enzyme. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 134: 135– 141. https://doi.org/10.1016/S1096-4959(02)00220-8

Bauchau AG 1980. Crustaceans. p. 385–420. In: Ratcliffe NA and Rowley AF (Eds.), Invertebrate Blood Cells, Vol. 2. New York, Academic Press.

Bouallegui YA 2021. Comprehensive review on crustaceans' immune system with a focus on freshwater crayfish in relation to crayfish plague disease. *Frontiers in Immunology*, 12: 667787. https://doi.org/10.3389/fimmu.2021.667787

Bruno D; Montali A; Gariboldi M; Wronska AK; Kaczmarek A, Mohamed A; Ling T; Casartelli M and Tettamanti G 2023. Morphofunctional characterization of hemocytes in black soldier fly larvae. *Insect Science*, 30(4): 912–932. https://doi.org/10.1111/1744-7917.13111

Carballal MJ; Lopez MC; Azevedo C and Villalba A 1997. Hemolymph cell types of the mussel *Mytilus galloprovincialis*. *Diseases of Aquatic Organisms*, 29: 127–135. https://doi.org/10.3354/dao029127

Cho SM and Jeong WG 2005. Spawning impact on lysosomal stability of the Pacific Oyster, *Crassostrea gigas*. *Aquaculture*, 244: 383–387. https://doi.org/10.1016/j.aquaculture.2004.12.013

Davey HM and Kell DB 1996. Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single-cell analyses. *Microbiological reviews*, 60(4): 641–696. https://doi.org/10.1128/mr.60.4.641-696.1996

Deyashi M and Chakraborty SB 2022. Cytomorphological characterization, classification and counting of haemocytes in freshwater crab, *Varuna litterata* (Crustacea: Decapoda). *Invertebrate Zoology*, 19: 120–134. https://doi.org/10.15298/invertzool.19.2.02

Donaghy L; Lambert C; Choi KS and Soudant P 2009. Hemocytes of the carpet shell clam (*Ruditapes decussatus*) and the Manila clam (*Ruditapes philippinarum*): Current knowledge and future prospects. *Aquaculture*, 297: 10–24. https://doi.org/10.1016/j.aquaculture.2009.09.003

Fiorotti J; Menna-Barreto RFS; Gôlo PS; Coutinho-Rodrigues CJB; Bitencourt ROB; Spadacci-Morena DD and Bittencourt VREP 2019. Ultrastructural and cytotoxic effects of *Metarhizium robertsii* infection on *Rhipicephalus microplus* hemocytes. *Frontiers in physiology*, 10: 654. https://doi.org/10.3389/fphys.2019.00654

Giulianini PG; Bertolo F; Battistella S and Amirante GA 2003. Ultrastructure of the hemocytes of *Cetonischema aeruginosa* larvae (Coleoptera, Scarabaeidae): involvement of both granulocytes and oenocytoids in in vivo phagocytosis. *Tissue Cell*, 35(4): 243–251. https://doi.org/10.1016/S0040-8166(03)00037-5

Hose JE; Martin GG and Gerard AS 1990. A decapod hemocyte classification scheme integrating morphology, cytochemistry, and function. *The Biological Bulletin*, 178: 33–45. https://doi.org/10.2307/1541535

Jia Z; Wang L; Jiang S; Sun M; Wang M; Yi Q and Song L 2017. Functional characterization of hemocytes from Chinese mitten crab *Eriocheir sinensis* by flow cytometry. *Fish Shellfish Immunology*, 69: 15–25. https://doi.org/10.1016/j.fsi.2017.08.001

Jiang S; Jia Z; Xin L; Sun Y; Zhang R; Wang W; Wang L and Song L 2016. The cytochemical and ultrastructural characteristics of phagocytes in the Pacific oyster *Crassostrea gigas*. *Fish Shellfish Immunology*, 55: 490–498. https://doi.org/10.1016/j.fsi.2016.06.024

Johansson MW; Keyser P; Sritunyalucksana K and Söderhäll K 2000. Crustacean haemocytes and haematopoiesis. *Aquaculture*, 191(1-3): 45–52. https://doi.org/10.1016/S0044-8486(00)00418-X

Latha PN; Raghavan SDA and Kenchappa CSB 2017. Ultrastructure of haemocytes of the freshwater crab *Travancoriana schirnerae*. *Turkish Journal of Fisheries and Aquatic Sciences*, (2): 231–243. https://doi.org/10.4194/1303-2712-v17_2_01

Laxmilatha P and Laxminarayana A 2004. Fine structure of the hemocytes of the Indian white shrimp, *Fenneropenaeus indicus* (H. Milne Edwards, 1837). *Crustaceana*, 77: 835–848. https://www.jstor.org/stable/20105763

Li F, Zheng Z, Li H, Fu R, Xu L and Yang F 2021. Crayfish hemocytes develop along the granular cell lineage. *Scientific Reports*, 11(1): 13099. https://doi.org/10.1038/s41598-021-92473-9

Liu S; Zheng SC; Li YL; Li J and Liu HP 2020. Hemocyte-mediated phagocytosis in crustaceans. *Frontiers in Immunology*, 11: 268. https://doi.org/10.3389/fimmu.2020.00268

Lu J; Shi Y; Yao T; Bai C; Jiang J and Ye L 2021. Gender differences in hemocyte immune parameters of Hong Kong oyster *Crassostrea hongkongensis* during immune stress. *Frontiers in Immunology*, 12: 659469. https://doi.org/10.3389/fimmu.2021.659469

Martin GG and Graves BL 1985. Fine structure and classification of shrimp hemocytes. *Journal of morphology*, 185: 339 –348. https://doi.org/10.1002/jmor.1051850306

Matozzo V and Marin MG 2010. The role of haemocytes from the crab *Carcinus aestuarii* (Crustacea, Decapoda) in immune responses: a first survey. *Fish Shellfish Immunology*, 28: 534–541. https://doi.org/10.1016/j.fsi.2009.12.003

Mengal K; Kor G; Kouba A; Kozák P and Niksirat H 2023. Hemocyte coagulation and phagocytic behavior in early stages of injury in crayfish (Arthropoda: Decapoda) affect their morphology. *Developmental and Comparative Immunology*, 141: 104618. https://doi.org/10.1016/j.dci.2022.104618

Picot S; Morga B; Faury N; Chollet B; Dégremont L; Travers MA; Renault T and Arzul I 2019. A study of autophagy in hemocytes of the Pacific oyster, *Crassostrea gigas*. *Autophagy*, 15(10): 1801–1809. https://doi.org/10.1080/15548627.2019.1596490

Rosales C 2017. Cellular and molecular mechanisms of insect immunity. p.179–212. In: Vonnie S (Ed.), Insect Physiology and Ecology, London, IntechOpen.

Salem HM; Hussein MA; Hafez SE; Hussein MA and Sayed RM 2014. Ultrastructure changes in the haemocytes of *Galleria mellonella* larvae treated with gamma irradiated *Steinernema carpocapsae* BA2. *Journal of Radiation Research and Applied Sciences*, 7(1): 74–79. https://doi.org/10.1016/j.jrras.2013.12.005

Schrand AM; Schlager JJ; Dai L and Hussain SM 2010. Preparation of cells for assessing ultrastructural localization of

nanoparticles with transmission electron microscopy. *Nature Protocols*, 5(4): 744-757. https://doi:10.1038/nprot.2010.2

Sharma PR; Sharma OP and Saxena BP 2008. Effect of sweet flag rhizome oil (*Acorus calamus*) on hemogram and ultrastructure of hemocytes of the tobacco armyworm, *Spodoptera litura* (Lepidoptera: Noctuidae). *Micron*, 39: 544–551. https://doi.org/10.1016/j.micron.2007.07.005

Shaurub ESH; Abd El-Meguid A and Abd El-Aziz N 2014. Quantificative and ultrastructural changes in the haemocytes of *Spodoptera littoralis* (Boisd.) treated individually or in combination with *Spodoptera littoralis* multicapside nucleopolyhedrovirus (SpliMNPV) and Azadirachtin. *Micron*, 65: 62–68. https://doi.org/10.1016/j.micron.2014.04.010

Söderhäll K; Smith VJ and Johansson MW 1986. Exocytosis and uptake of bacteria by isolated hemocyte populations of two crustaceans: evidence for cellular cooperation in the defence reactions of arthropods. *Cell and Tissue Research*, 245: 43–49. https://doi.org/10.1007/BF00218085

Tojo S; Naganuma F; Arakawa k and Yokoo S 2000. Involvement of both granular cells and plasmatocytes in phagocytic reactions in the greater wax moth, *Galleria mellonella*. *Journal of Insect Physiology*, 46: 1129-1135. https://doi.org/10.1016/s0022-1910(99)00223-1

Vacca LL and Fingerman M 1983. The roles of hemocytes in tanning during the molting cycle: A histochemical study of the fiddler crab, *Uca pugilator*. *The Biological Bulletin*, 165: 758–777. https://doi.org/10.2307/1541477

Vogan CL and Rowley AF 2002. Effects of shell disease syndrome on the hemocytes and humoral defences of the edible crab, *Cancer pagurus*. *Aquaculture*, 205: 237–252. https://doi.org/10.1016/S0044-8486(01)00703-7

Wang W; Li M; Wang L; Chen H; Liu Z; Jia Z; Qiu L and Song L 2017. The granulocytes are the main immunocompetent hemocytes in *Crassostrea gigas*. *Developmental and Comparative Immunology*, 67: 221–228. https://doi.org/10.1016/j.dci.2016.09.017

Wang Y; Hu M; Chiang MWL; Shin PKS and Cheung SG 2012. Characterization of subpopulations and immune-related parameters of hemocytes in the green-lipped mussel *Perna viridis*. *Fish and shellfish immunology*, 32(3): 381–390. https://doi.org/10.1016/j.fsi.2011.08.024

Wang YT; Liu W; Seah JN; Lam CS; Xiang JH; Korzh V and Kwang J 2002. White spot syndrome virus (WSSV) infects specific hemocytes of the shrimp *Penaeus merguiensis*. *Diseases of aquatic organisms*, 52(3): 249–259. https://doi.org/10.3354/dao052249

Wentao Z; Wen L; Yunlong Z; Danli W; Zhongxiang M and Getao S 2017. Ultrastructural and immunocytochemical analysis of circulating hemocytes from *Cherax quadricarinatus* (von Martens, 1868). *Indian Journal of Animal Research*, 51: 129–134. https://doi.org/10.18805/ijar.v0iOF.6823

Wu F; Xie Z; Yan M; Li Q; Song J; Hu M and Wang Y 2019. Classification and characterization of hemocytes from two Asian horseshoe crab species *Tachypleus tridentatus* and *Carcinoscorpius rotundicauda*. *Scientific Reports*, 9(1): 7095. https://doi.org/10.1038/s41598-019-43630-8

Wu H; Li Y; Lang X and Wang L 2015. Bioaccumulation, morphological changes, and induction of metallothionein gene expression in the digestive system of the freshwater crab *Sinopotamon henanense* after exposure to cadmium. *Environmental Science and Pollution Research*, 22: 11585–11594. https://doi.org/10.1007/s11356-015-4419-5

Zhou YL; Gu WB; Tu DD; Zhu QH; Zhou ZK; Chen YY and Shu MA 2018. Hemocytes of the mud crab *Scylla paramamosain*: Cytometric, morphological characterization and involvement in immune responses. *Fish and shellfish immunology*, 72: 459–469. https://doi.org/10.1016/j.fsi.2017.10.055

ADDITIONAL INFORMATION AND DECLARATIONS

Author Contributions

Conceptualization and Design: MKS. Performed research: PVAK. Acquisition of data: PVAK, MKS. Analysis and interpretation of data: PVAK, MKS. Preparation of figures/tables/maps: PVAK, MKS. Writing - original draft: MKS, PVAK. Writing -critical review & editing: MKS.

Consent for publication

All authors declare that they have reviewed the content of the manuscript and gave their consent to submit the document.

Competing interests

The authors declare no competing interest.

Data availability

All study data are included in the article and/or supplementary material.

Funding and grant disclosures

There were no external funding sources for this study.

Study association

This work is a part of the Master dissertation of PVAK in the program Applied Zoology, Department of Zoology, Kannur University, Kerala, India.